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Molecular data reveal an undescribed cryptic species of *Costasiella* Pruvot-Fol, 1951 (Euthyneura: Sacoglossa: Limapontidae) in the Bahamas

Erika Espinoza[1], Anne DuPont[2], and Ángel Valdés[1]

[1]Department of Biological Sciences, California State Polytechnic University, 3801 West Temple Avenue, Pomona, California 91768, U.S.A.
[2]4070 NW 7th Lane, Delray Beach, Florida 33445, U.S.A.
Correspondence, Ángel Valdés: aavaldes@csupomona.edu

Abstract: Molecular and morphological evidence revealed the existence of two cryptic species of *Costasiella* Pruvot-Fol, 1951 in the Bahamas. A review of the literature indicates that one of these species is the western Atlantic widespread species *Costasiella ocellifera* (Simroth, 1895), whereas the other species is undescribed. The new species is externally similar to *Costasiella ocellifera* but can be distinguished by the absence of a penial stylet and the radular morphology. Phylogenetic and species delimitation analyses confirm that the new species is genetically distinct from *C. ocellifera*. The new species has only been found in the Bahamas, but there are possible records from Cuba and Jamaica.

Key words: cryptic species, mtDNA, nDNA, phylogenetics, species delimitation

Costasiella Pruvot-Fol, 1951 is a small group of sacoglossan sea slugs (Limapontidae Gray, 1847) consisting of 14 valid species (Swennen 2007). *Costasiella* is particularly species rich in the Indo-Pacific tropics and subtropics, with six species described from the Ryukyu Islands alone (Ichikawa 1993), and one from each of the following localities: Japan (Baba 1959, 1961), Micronesia (Marcus 1982), Hong Kong (Jensen 1985), Australia (Jensen 1997), and Thailand (Swennen 2007).

In the Atlantic Ocean, *Costasiella* is represented by a Mediterranean species, *Costasiella virescens* Pruvot-Fol, 1951 and two western Atlantic species, *C. ocellifera* (Simroth, 1895) and *C. nonatoi* Ev. Marcus and Er. Marcus, 1960. A third western Atlantic species described from Brazil, *C. lilianae* (Ev. Marcus and Er. Marcus, 1969) is considered a junior synonym of *C. ocellifera* (see Clark 1984).

In this paper we describe a third western Atlantic species of *Costasiella* collected from the Bahamas. This species has been reported in the literature as a color variation of *C. ocellifera* (see Redfern 2001). However, molecular and morphological data revealed that it is a distinct species.

MATERIAL AND METHODS

Source of specimens

Thirteen specimens initially identified as *Costasiella ocellifera*, two of which have been re-identified as a new cryptic species were sequenced for this study (Table 1). Thirty-two additional specimens were examined morphologically but not sequenced. All specimens were collected in Stocking Island, Exumas, Bahamas. Additional sequences were obtained from Patrick Krug and GenBank. Specimens were photographed alive, narcotized using a 1M solution of $MgCl_2$, and preserved in 70% EtOH. All the non-type specimens collected are deposited at the Natural History Museum of Los Angeles County (LACM) and the invertebrate collections of the California State Polytechnic University (CPIC). Type material is deposited exclusively at the LACM. Egg masses were obtained from individuals isolated in plastic containers and photographed in the field.

Morphological examination

Two specimens of each species were dissected to remove the pharynx. For each specimen tissue surrounding the radula was dissolved by leaving the pharynx submerged in 10% NaOH overnight. The radulae were rinsed in distilled water, dried, mounted, and sputter coated for examination with a scanning electron microscope (SEM) Hitachi S-3000N at the LACM. Several specimens of *Costasiella ocellifera* and one of the new species were dissected under a Nikon SMZ-1000 dissecting microscope with a camera lucida attachment to investigate the morphology of the reproductive system. The penises of two specimens were removed and photographed with an Olympus CX-31 compound microscope.

DNA extraction

DNA extraction was performed using a hot Chelex® protocol. Approximately 1–3 mg of the foot was cut into fine pieces for extraction. For the Chelex® extraction, the foot tissue was rinsed and rehydrated using 1.0 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 min. A 10% (w/v) Chelex® 100 (100–200 mesh, sodium form, Bio-Rad) solution was prepared using TE buffer. After rehydration, the

Table 1. Material used in the phylogenetic analysis, including locality information, collection date, voucher numbers, and GenBank accession numbers. An asterisk denotes the outgroup.

				GenBank Accession Numbers		
Species	Locality	Collection date	Voucher number	16S	COI	H3
C. ocellifera	Stocking Island, Bahamas	Dec 15, 2007	CPIC 00019	KF411384	-	KF411405
C. ocellifera	Stocking Island, Bahamas	Feb 16, 2009	CPIC 00096	KF411385	KF411397	KF411406
C. ocellifera	Stocking Island, Bahamas	Feb 16, 2009	CPIC 00097	KF411386	-	KF411407
C. ocellifera	Stocking Island, Bahamas	Feb 16, 2009	CPIC 00098	KF411387	KF411398	KF411408
C. ocellifera	Stocking Island, Bahamas	Jan 06, 2009	CPIC 00099	KF411388	-	KF411409
C. ocellifera	Stocking Island, Bahamas	Dec 26, 2009	CPIC 00252	KF411389	KF411399	KF411410
C. ocellifera	Stocking Island, Bahamas	Jan 26, 2010	CPIC 00267	KF411390	KF411400	KF411411
C. ocellifera	Stocking Island, Bahamas	Dec 28, 2009	CPIC 00283	KF411391	-	KF411412
C. ocellifera	Stocking Island, Bahamas	Dec 28, 2009	CPIC 00284	KF411392	KF411401	KF411413
C. ocellifera	Stocking Island, Bahamas	Dec 28, 2008	CPIC 00285	KF411393	KF411402	KF411414
C. ocellifera	Stocking Island, Bahamas	Dec 31, 2008	CPIC 00100	KF411394	KF411403	KF411415
C. ocellifera	St. George, Bermuda	Jun 6, 2006	-	KF438670	KF438672	KF438674
C. ocellifera	Key West, Florida	Oct, 2006	-	KF438669	KF438671	KF438673
C. ocellifera	Bahamas	-	-	DQ480216	DQ471253	DQ534806
*C. kuroshimae**	Guam	-	-	DQ480215	DQ471252	DQ534805
C. nonatoi	Bocas del Toro, Panama	2005	-	GU191037	GU191065	-
C. patricki	Stocking Island, Bahamas	Dec 15, 2007	LACM 3241	KF411395	KF411404	KF411416
C. patricki	Stocking Island, Bahamas	Dec 31, 2008	LACM 3242	KF411396	-	KF411417

tissue mixture was then centrifuged, 975.00 mL of the supernatant was removed, and 175.00 mL of the Chelex® solution was added. Samples were then heated in a 56 °C water bath for 20 min, heated in a 100 °C heating block for 8 min, and the supernatant was then used for PCR.

PCR amplification and sequencing

Histone-3 universal primers (H3AF 5′-ATGGCTCG TACCAAGCAGACGGC-3′, H3 AR 5′-ATATCCTTGGG CATGATGGTGAC-3′) developed by Colgan *et al.* (1998), 16S rRNA universal primers (16S ar-L 5′-CGCCTGTTTAT CAAAAACAT-3′, 16S br-H 5′-CCGGTCTGAACTCAGAT CACGT-3′) developed by Palumbi (1996), COI universal primers (LCO1490 5′-GGTCAACAAATCATAAAGATATT GG-3′, HCO2198 5′-TAAACTTCAGGGTGACCAAAAAAT CA-3′) developed by Folmer *et al.* (1994) were used to amplify the regions of interest for most specimens. The opisthobranch-specific COI primers (NAF-COI 5′-GCC TTTTCAACAAACCATAAAGA-3′, NAR-COI 5′-CCATCCT GGTAAAATTAAAATATA-3′) developed by Ornelas-Gatdula *et al.* (2011) were used to amplify the COI fragment in the new species.

The master mix was prepared using 34.75 µL H_2O, 5.00 µL Buffer B (ExACTGene, Fisher Scientific), 5.00 µL 25 mM $MgCl_2$, 1.00 µL 40mM dNTPs, 1.00 µL 10mM primer 1, 1.00 µL 10mM primer 2, 0.25 µL 5 mg/mL Taq, and 2.00 µL extracted DNA. The polymerase chain reactions (PCR) were run on an Eppendorf Mastercycler personal thermocycler. Reaction conditions for H3 (universal) and 16S (universal) were as follows: heated lid at 105 °C and initial denaturation of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, followed by a final elongation of 72 °C for 7 min. Reaction conditions for COI (universal) were as follows: heated lid at 105 °C and initial denaturation of 95 °C for 3 min, 35 cycles of 94 °C for 45 s, 45 °C for 45 s, and 72 °C for 2 min, followed by a final elongation step of 72 °C for 10 min. NAF-COI and NAR-COI external primers (used for specimen LACM 3241) had the same reaction as the COI (universal) primers, except for the number of cycles which was changed to 39 cycles.

PCR products yielding bands of appropriate size (approximately 395 bp for H3, 495 bp for 16S, and 750 bp for COI) were purified using the Montage PCR Cleanup Kit (Millipore). Cleaned PCR samples were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Each primer was diluted to 4.0 pmol/µL to send out for sequencing with the PCR products. PCR products were diluted to 6.0, 7.5, and 11.5ng/µL for H3, 16S, and COI respectively. Samples were sequenced at the City of Hope DNA Sequencing Laboratory (Duarte, CA) using chemistry types BigDye V1.1 for fragments less than 500 bp and BigDye V3.1 for fragments larger than 500 bp. One specimen (LACM 3241) was sequenced at the Eton Bioscience, Inc. (San Diego, CA).

Molecular analyses

Sequences for each gene were assembled and edited using Geneious Pro 4.7.4 (Drummond *et al.* 2010). Geneious was also used to extract the consensus sequence between the primer regions, to construct the alignment for each gene using the default parameters, and to concatenate the alignments. Analyses were conducted with trimmed and untrimmed alignments with no changes in the final results. The total length of the aligned sequences ranged between 305–328 bp for H3, 440–454 bp for 16S, and 501–658 bp for COI.

To assess whether H3, 16S, and COI have significantly conflicting signals the incongruence length difference (ILD) test (Mickevich and Farris 1981, Farris *et al.* 1994), implemented in PAUP*4.0 as the partition homogeneity test (Swofford 2002), was conducted for all genes combined.

The levels of saturation for each gene and for the first and second versus third codon positions of COI and H3 were investigated using the substitution saturation test developed by Xia *et al.* (2003) and Xia and Lemey (2009) implemented in the program DAMBE (Xia and Xie 2001). *Costasiella kuroshimae* Ichikawa, 1993 was selected as the outgroup and *Costasiella nonatoi* was included for comparison with *Costasiella ocellifera*. Since *C. nonatoi* and *C. ocellifera* are sympatric it is assumed that they are more closely related than the Japanese species *C. kuroshimae*.

The Akaike information criterion (Akaike 1974) was executed in MrModeltest (Nylander 2004) to determine the best-fit model of evolution. MrModeltest selected the models GTR+G for CO1, GTR+I+G for 16S, and HKY+I for H3 (CO1 γ shape = 0.1446; 16S γ shape = 0.4241, proportion of invariant sites = 0.3832; H3 proportion of invariant sites = 0.8899). Bayesian analyses were executed in MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001), partitioned by gene (unlinked). The Markov chain Monte Carlo analysis was run with two runs of six chains for ten million generations, with sampling every 100 generations. Effective sample sizes and convergence of runs were assessed using Tracer 1.4.1 (Rambaut and Drummond 2007). The default 25% burn-in was applied before constructing majority-rule consensus tree(s).

Diagnostic nucleotides for each clade were identified visually in the alignments after collapsing identical haplotypes using the program Collapse 1.2 (Posada 2004). The Species Delimitation plugin (Masters *et al.* 2011) for Geneious was used to provide a statistical framework to help determine whether clades obtained in the phylogenetic analysis have identity as distinct species. The statistics implemented were the ratio between the mean distance within the members of the clade and the mean distance of those individuals to the nearest clade and the p ID, which represents the mean probability, 95% confidence interval, for a member of the putative species to fit inside (strict p ID), or at least to be the sister group (liberal p ID) of the clade made up by the other individuals belonging to this species (Table 2). Additionally, the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al.* 2012) was implemented to compute the theoretical maximal limit of the intraspecific diversity (using a coalescent model) with COI and 16S. ABGD identifies in the entire distribution of pairwise distances which gap (superior to the maximal limit of the intraspecific diversity), potentially corresponds to the so-called 'Barcoding gap,' a hypothetical limit between intra- and interspecific diversity (Puillandre *et al.* 2012). Inference of the limit and gap detection are then recursively applied to previously obtained groups to get finer partitions until there is no further partitioning. The online version of the software (http://wwwabi.snv.jussieu.fr/public/abgd/) was used to analyze the COI and 16S datasets. MEGA 4.0 (Tamura *et al.* 2007) was used to build the distance matrices for COI and 16S using a Tamura Nei model. The data was analyzed using the two available models: Jukes-Cantor (JC69) and Kimura (K80). The program requires two user-specified values: P (prior limit to intraspecific diversity) and X (proxy for minimum gap width). To evaluate the effect on the datasets X values from 0.1 to 5 were tested and the maximum Pmax value was extended from 0.1 to 0.2.

RESULTS

The saturation analysis showed insignificant levels of saturation for all three genes, 16S (Iss = 0.1765 < Iss.c = 0.7062, $P = 0.0000$), COI (Iss = 0.2620 < Iss.c = 0.7385, $P = 0.0000$), and H3 (Iss = 0.0341 < Iss.c = 0.6755, $P = 0.0000$). The ILD test showed non-significant conflicting signals between the genes combined: CO1 vs. H3 ($P = 1.00$), 16S vs. H3 ($P = 0.99$), and CO1 vs. 16S ($P = 1.00$).

The combined analysis of the three genes (H3, 16S, and CO1) produced a consensus Bayesian tree (Fig. 1) in which

Table 2. Species delimitation results from the Bayesian tree.

Species	Closest Species	Monophyly	Intra dist	Inter dist closest	Intra/Inter	P ID(strict)	P ID(liberal)
C. nonatoi	*C. ocellifera*	Yes	0.000	0.612	0.00	0.0	0.96
C. ocellifera	*C. patricki*	Yes	0.018	0.175	0.11	0.94	0.98
C. patricki	*C. ocellifera*	Yes	0.007	0.175	0.04	0.57	0.96

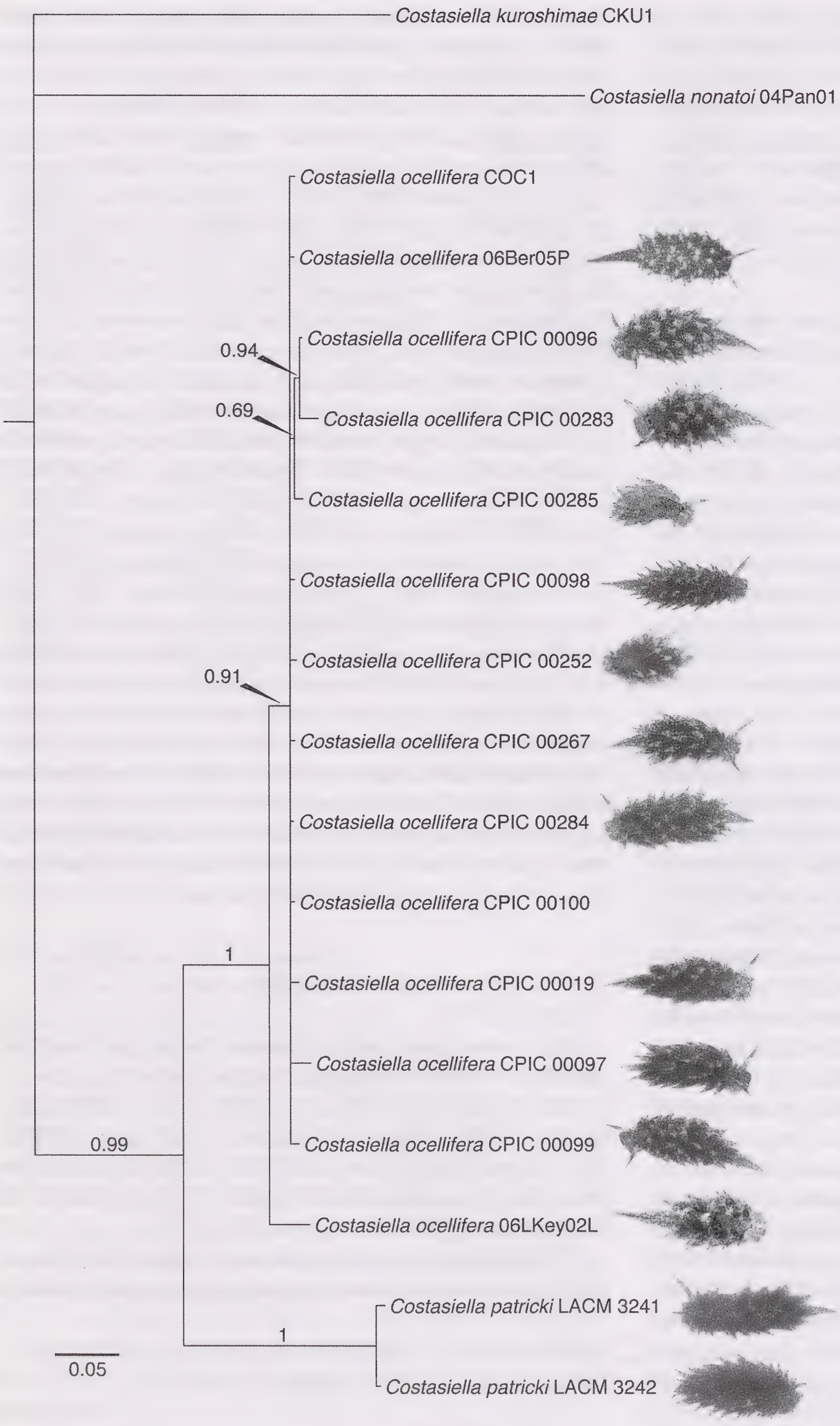

Figure 1. Bayesian consensus tree of the specimens examined with posterior probability values and illustrations of the specimens from which sequences were obtained.

the monophyly of *Costasiella ocellifera* is well supported (pp = 1), including specimens from the Bahamas, Bermuda and the Florida Keys. However, two specimens from the Bahamas form another monophyletic group (pp = 1), sister to *C. ocellifera* (pp = 0.99). These two specimens are herein referred to as *Costasiella patricki*, new species.

The species delimitation analysis (Table 2) for *Costasiella ocellifera* and *C. patricki* shows that values of the ratio between the average distance between the samples of one group (Intra Dist) and the average distance between those samples and the closest clade (Inter Dist) are small, below 0.25. This means that genetic differences within all clades are small relative to the differences between clades, so that there is a well-defined separation of clades. The probability (p ID) of a new sequence fitting inside (strict) or at least as sister group (liberal) of its clade is high in all cases. The results for *Costasiella nonatoi* are not discussed since only one specimen of this species was included and, therefore, meaningful analyses are not possible.

The ABGD analysis with the standard settings recovered 2 groups for COI and 16S respectively. These groups correspond to the clades recovered in the phylogenetic analyses (*Costasiella ocellifera* and *Costasiella patricki*) providing further support for the taxonomic decisions. ABGD grouping results were independent from the chosen model (Jukes-Cantor and Kimura) and unaffected by changes of prior limit of intraspecific variation or X-value.

Because of the genetic differences between specimens of *Costasiella ocellifera* and *Costasiella patricki*, the latter is formally described as a distinct species in systematics descriptions section below and compared morphologically with *C. ocellifera*.

SYSTEMATICS DESCRIPTIONS

Costasiella ocellifera (Simroth, 1895)

(Figs. 2A, 3A, 4A, 5A)

Doto ocellifera Simroth, 1895: Simroth 1895: 168–170, pl. 20, figs. 6–10.

Costasiella ocellifera (Simroth, 1895): Clark 1984: 92–33, fig. 32.

Stiliger lilianae Ev. Marcus and Er. Marcus, 1969: Marcus and Marcus 1969: 7–12, figs. 22–28.

Ercolania lilianae (Ev. Marcus and Er. Marcus, 1969): Marcus 1977: 7.

Costasiella lilianae (Ev. Marcus and Er. Marcus, 1969): Thompson 1977: 137–138, figs. 26j, 32a.

Material examined

Stocking Island, Bahamas (23.5333°N, 75.7667°W), between 1–3 m depth, 18 February 2005, 6 specimens (LACM 172285); 18 February 2005, 6 specimens (LACM 172286); 12 December 2005, 1 specimen (LACM 173222); 17 December 2005, 1 specimen (LACM 173223); 3 February 2007, 5 specimens, dissected (CPIC 00021); 3 February 2007, 5 specimens (CPIC 00022); 15 December 2007, 4 specimens (CPIC 00019) (1 specimen sequenced, isolate EE70), 31 December 2008, 1 specimen (CPIC 00100) (isolate EE88); 6 January 2009, 5 specimens (CPIC 00099) (1 specimen sequenced, isolate EE77); 26 January 2009, 2 specimens (CPIC 00267) (1 specimen sequenced, isolate EE81); 16 February 2009, 1 specimen (CPIC 00096) (isolate EE74); 16 February 2009, 1 specimen (CPIC 00097) (isolate EE75); 16 February 2009, 1 specimen (CPIC 00098) (isolate EE76); 26 December 2009, 3 specimens (CPIC 00252) (1 specimen sequenced, isolate EE80); 28 December 2009, 1 specimen (CPIC 00283) (isolate EE82); 28 December 2009, 1 specimen (CPIC 00284) (isolate EE83); 28 December 2009, 1 specimen (CPIC 00285) (isolate EE84).

External morphology

Background translucent grayish white, with large orange patch on the head, running from the anterior end of the body to the pericardium, between the rhinophores (Figs. 2A). Body covered with dark brown or black spots, varying in density among specimens. Cerata variable in color, from yellow in some juvenile specimens to dark green, covered with numerous opaque white spots of varying sizes in the green animals, and black spots in the yellow animals. Each ceras has a large light blue spot. A sub-apical yellow to orange ring occupies approximately 1/4 to 1/7 of the length of each ceras, followed by a white ring and a black tip. Rhinophores translucent, with numerous dark spots more densely concentrated mid-length, forming a dark band. A bright pale blue spot surrounded by a black ring is located between the eyes and the pericardium.

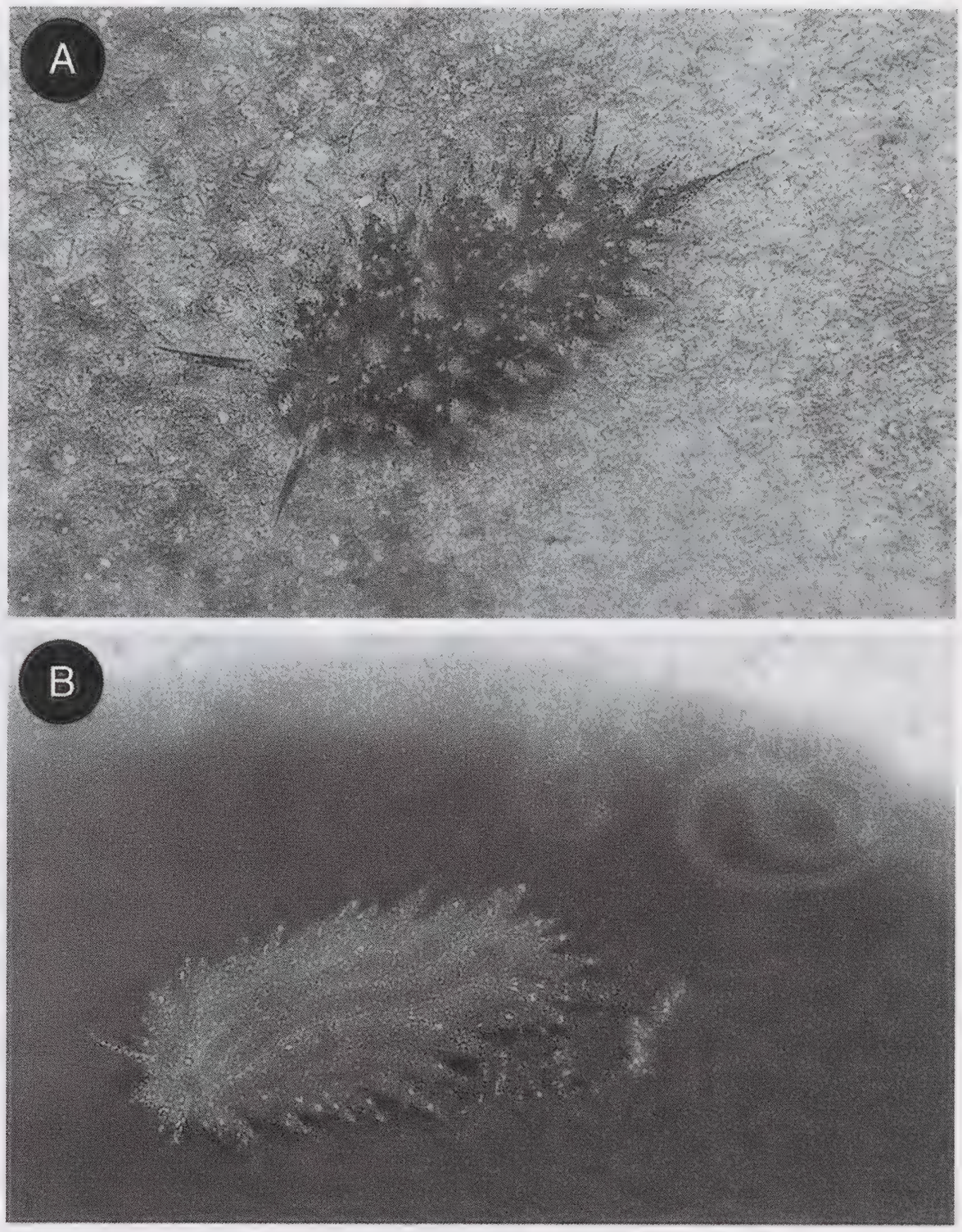

Figure 2. Live animals on *Avrainvillea* photographed in Stocking Island, Bahamas. **A,** *Costasiella ocellifera*, 12 December 2005, and **B,** *Costasiella patricki* n. sp., 18 February 2005, there is no direct evidence the two eggs masses in the photo were deposited by the specimen illustrated.

Body elongate, covered with numerous cerata. Cerata inflated, narrow at the base and wider mid-length, narrowing again apically into a very long and narrow apex. Head almost square, with two inconspicuous and blunt anterior extensions. Rhinophores elongate, smooth, narrow. Anterior end of the foot with two blunt, lateral expansions. Foot sole divided into a longer and wider anterior portion that occupies 2/3 of the length of the body and a shorter and narrower posterior section. Posterior end of the body triangular, lacking ramifications of the digestive gland, distally ending in a narrow tip. Eyes large, close together, situated between the rhinophores. Anus located on the anterior right hand side of the pericardium.

Penial morphology

The penis is elongate connected to a relatively long and convoluted deferent duct, and having a hollow penial stylet on the tip (Figs. 3A, 4A).

Radular morphology

Radular with 23 teeth (CPIC 00099: 7 mm long), 9 in the descending limb and 14 in the ascending limb (Fig. 5A). Teeth

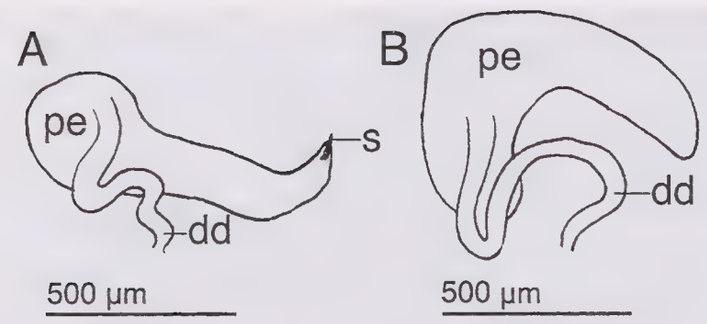

Figure 3. Drawings of the penial morphology. **A,** *Costasiella ocellifera* (CPIC 00021), and **B,** *Costasiella patricki* n. sp. (CPIC 00020). Abbreviations: **dd,** deferent duct; **pe,** penis; **s,** penial stylet.

elongate, with sharp tips, and lacking denticles. Teeth base short, concave at the base, followed by a narrowing of the tooth, a widening, another narrowing and a widening into the apical end.

Biology

Costasiella ocellifera feeds on the green alga *Avrainvillea longicaulis* (see Cimino and Ghiselin 2009) and *A. nigricans* (see Redfern 2001). It contains a brominated diphenylmethane derivative, avrainvilleol that has been shown experimentally to deter feeding by fish (Hay *et al.* 1990). This species displays poecilogony, with intracapsular metamorphosis and planktonic larvae. The developmental mode varies at the population level and appears to be fixed over the lifetime of an individual (Ellingson 2006). In the Bahamas we observed egg masses with similar egg sizes (Fig. 6A–C) suggesting only one developmental mode is found in this population. The egg mass is a ribbon of eggs forming a spiral with 1.5–2.5 whorls (Fig. 6A–C). The eggs are arranged in transverse rows of 4–6 eggs. Some egg masses are more tightly packed and coiled, and have more whorls (Fig. 6A, C) than others (Fig. 6B).

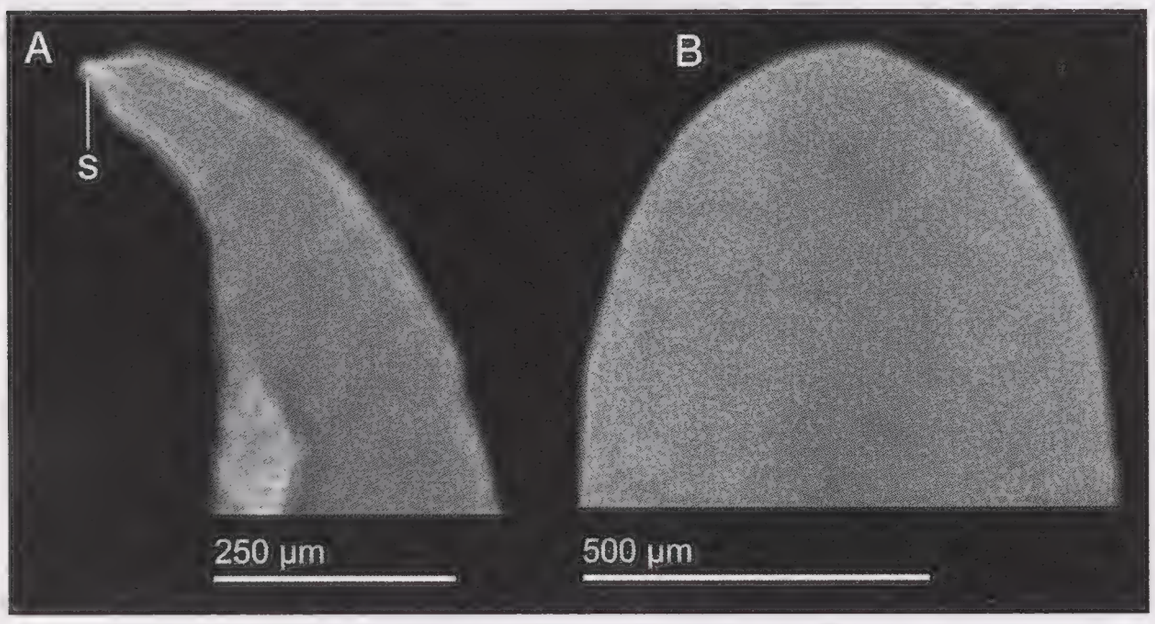

Figure 4. Photographs of the distal end of the penis under a compound microscope. Because of pressure from the coverslip the penises are artificially deformed. **A,** *Costasiella ocellifera* (CPIC 00021), and **B,** *Costasiella patricki* n. sp. (CPIC 00020). Abbreviation: **s,** penial stylet.

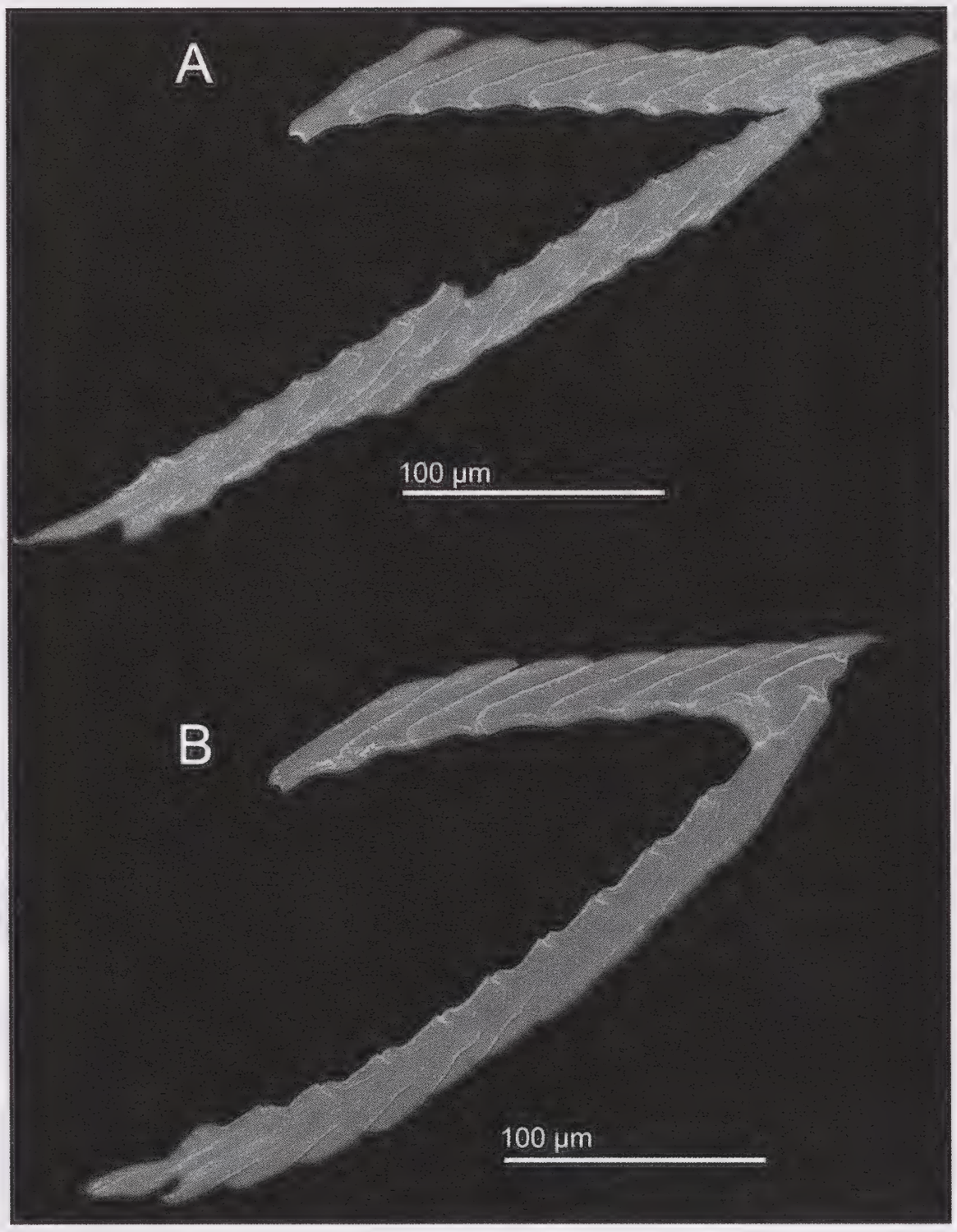

Figure 5. Scanning electron micrographs of the radulae. **A,** *Costasiella ocellifera* from Bahamas (CPIC 00099), and **B,** *Costasiella patricki* n. sp., paratype (LACM 3242).

***Costasiella patricki* new species**

(Figs. 2B, 3B, 4B, 5B)

Costasiella ocellifera (Simroth, 1895): Redfern 2001: fig 681B.

Type material

Holotype: Stocking Island, Bahamas (23.5333°N, 75.7667°W), 2 m depth, 15 December 2007, 8 mm long, preserved in 70% ethanol (LACM 3241) (isolate EE90).

Paratype: Stocking Island, Bahamas (23.5333°N, 75.7667°W), 2 m depth, 31 December 2008, 1 specimen 5 mm long, pharynx dissected, preserved in 70% ethanol (LACM 3242) (isolate EE91).

Additional specimens

Stocking Island, Bahamas (23.5333°N, 75.7667°W), 3 February 2007, 4 specimens, destroyed for dissection (CPIC 00020).

External morphology

Background orange, covered with numerous black spots and some opaque white patches (Fig. 2B). Cerata pale to dark

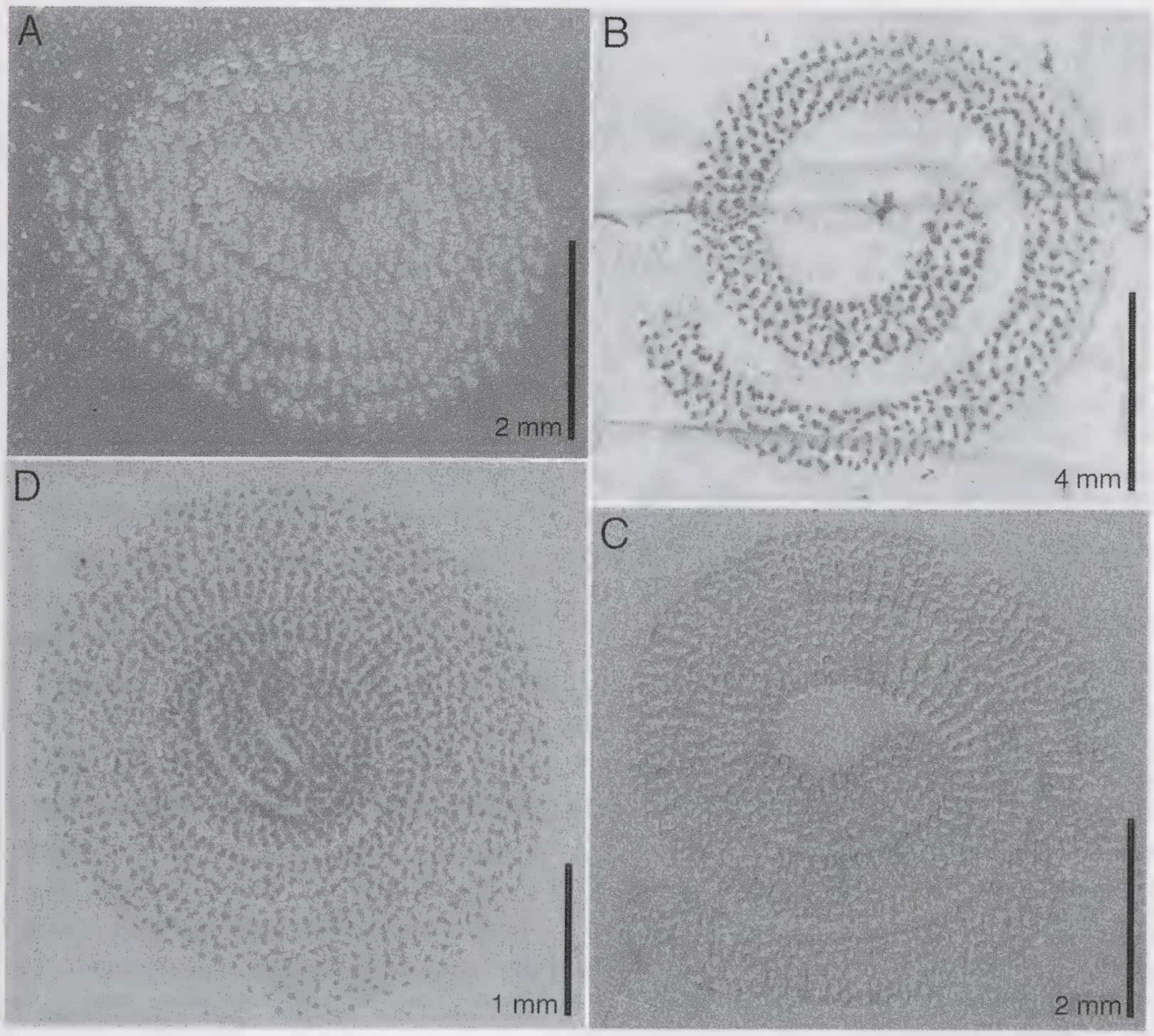

Figure 6. Photographs of egg masses. **A,** *Costasiella ocellifera*, 18 February 2005, **B,** *C. ocellifera*, 18 February 2005, **C,** *C. ocellifera*, 18 February 2005, and **D,** *C. patricki* n. sp., 18 February 2005.

green, covered with black dots, each bearing a large light blue spot. A sub-apical yellow to orange ring occupies approximately 1/8 to 1/10 of the length of each ceras, surrounded by a translucent white area proximally, and white tip with black dots distally. Rhinophores translucent, with numerous dark spots and an orange area mid-length. A bright pale blue spot surrounded by a black ring is located between the eyes and the pericardium.

Body elongate, covered with numerous cerata. Cerata inflated, narrow at the base and wider mid-length, narrowing again apically into a long and narrow apex. Head with two blunt anterior extensions clearly visible. Rhinophores elongate, smooth, narrow. Anterior end of the foot with two blunt, lateral expansions. Foot sole divided into a longer and wider anterior portion that occupies 2/3 of the length of the body and a shorter and narrower posterior section. Posterior end of the body triangular, lacking ramifications of the digestive gland, distally ending in a narrow tip. Eyes large, close together, situated between the rhinophores. Anus located on the anterior right hand side of the pericardium.

Penial morphology

The penis is wide, connected to a relatively long and simple deferent duct and devoid of armature (Figs. 3B, 4B).

Radular morphology

Radular with 19 teeth (LACM 3242: 5 mm long), 8 in the descending limb and 11 in the ascending limb (Fig. 5B). Teeth elongate, with sharp tips, and lacking denticles. Teeth base wide, concave at the base. Teeth blade with a constant width except for the apex, which narrows into a sharp, thin tip.

Biology

Costasiella patricki n. sp. is found on the green algae *Avrainvillea* spp. often together with *Costasiella ocellifera*. Only one egg mass of this species was observed (Fig. 6D). The egg mass is a ribbon of eggs forming a spiral with 4.5 whorls (Fig. 6D). The eggs are arranged in transverse rows of 6–7 eggs. Although the eggs were not measured with precision tools, they appear to be small suggesting this species could have planktonic larvae.

Etymology

This species is named after our colleague Patrick Krug, who has worked extensively on the systematics and ecology of Caribbean sacoglossans. He also provided nuclear and mitochondrial DNA sequences of two specimens of *Costasiella ocellifera* that were essential for the molecular analysis of this study.

DISCUSSION

Costasiella ocellifera and *Costasiella patricki* n. sp. are genetically and morphologically distinct and, therefore, are treated as different species. The presence of unique diagnostic characters in each species as well as the results of the species delimitation analysis confirmed their distinctiveness. However, the external morphological differences only became obvious when sequence data became available. The two species are very similar and often difficult to distinguish. Both *C. ocellifera* and *C. patricki* n. sp. often have a greenish appearance (because of the green cerata), although some juvenile

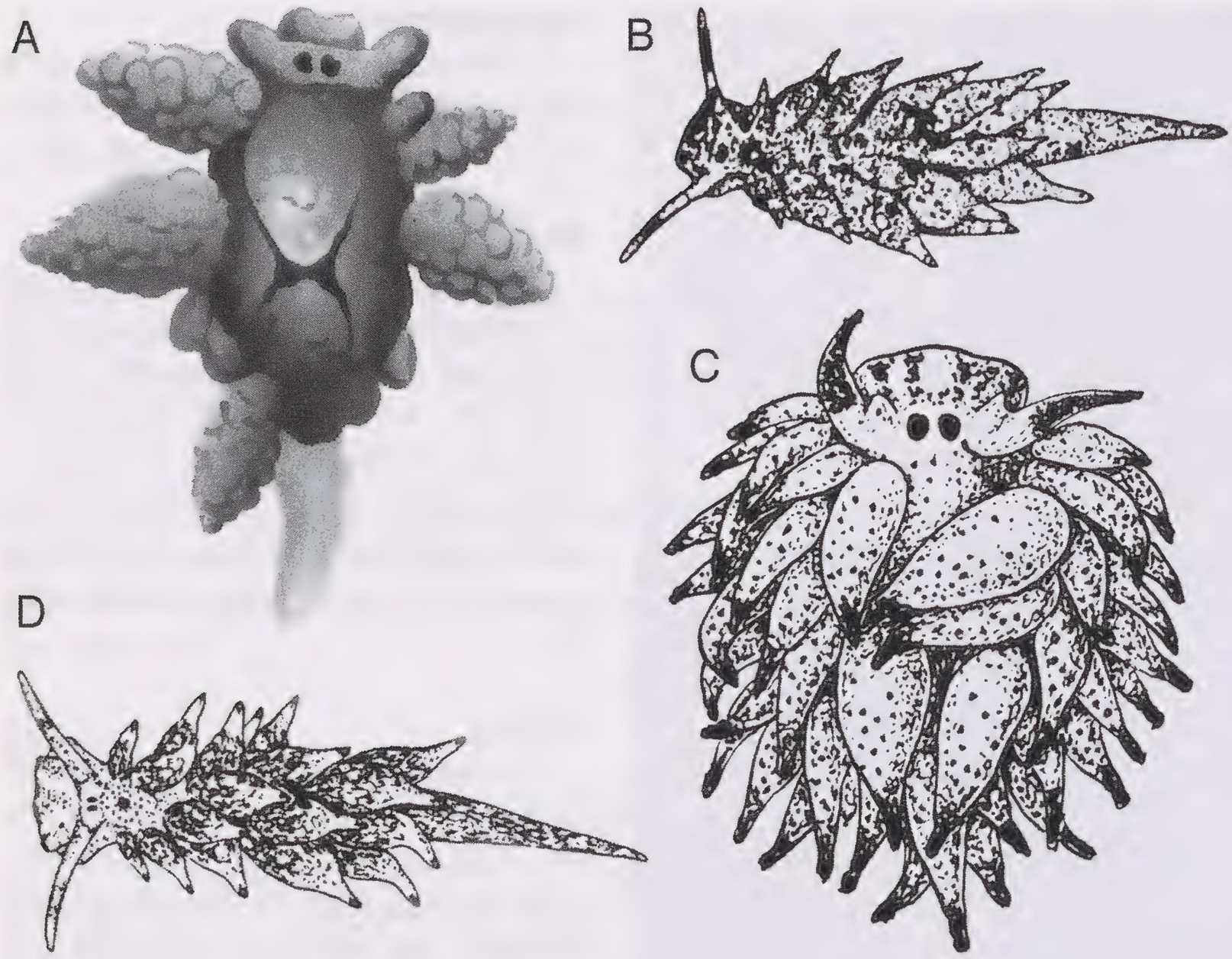

Figure 7. Reproductions of published illustrations of western Atlantic *Costasiella*. **A,** Original description of *Doto ocellifera* (from Simroth 1895), **B,** Redescription of *Costasiella ocellifera* (from Clark 1984), **C,** Original description of *Stiliger lilianae* (from Marcus and Marcus 1969), and **D,** Redescription of *Costasiella lilianae* (from Thompson 1977).

specimens of *C. ocellifera* may appear yellowish. Also, both species have a conspicuous blue spot behind the rhinophores and anterior of the pericardium. The main external difference between the two species is that *C. patricki* n. sp. is often lighter in color and the orange rings on the cerata are smaller, less conspicuous.

The internal morphological differences between the two species are more obvious, and include the absence of a penial stylet in *Costasiella patricki* n. sp., which is present in *Costasiella ocellifera*. The specimens dissected were very small, thus it was impossible to reliably describe other internal organs with gross anatomical examination.

Costasiella ocellifera and *Costasiella patricki* n. sp. also have different egg masses, having as many as 4 whorls in *C. patricki* n. sp. and only 1.5–2.5 whorls in *C. ocellifera*. However, because egg mass morphology, egg size and development is variable in *C. ocellifera*, this is probably not a reliable trait to distinguish the two species.

A review of the literature described below revealed that there is no available name for *Costasiella patricki* n. sp. and, therefore, is here described as a new species. The taxonomy of western Atlantic *Costasiella* has been problematic with two species names introduced for *C. ocellifera* and controversies regarding the number of valid species as well as their generic allocation. The first comprehensive description of a western Atlantic *Costasiella* was by Marcus and Marcus (1969), who described *Stiliger lilianae* from southern Brazil including detailed illustrations of the preserved animals (Fig. 7C). Baba and Hamatani (1970) suggested that this species should be transferred to *Costasiella*, but Marcus (1976) disagreed and included it in *Ercolania* Trinchese, 1872, based on the rhinophoral morphology. *Stiliger lilianae* was reported from Puerto Rico (Marcus 1976), Florida (Marcus 1977), and Jamaica (Thompson 1977). Thompson (1977) agreed with Baba and Hamatani (1970) including *Stiliger lilianae* in *Costasiella* because of the location of the eyes, close together between the rhinophores (Fig. 7D). The original description of *Stiliger lilianae* (Marcus and Marcus 1969) is very complete. It includes detailed anatomical descriptions and drawings of the reproductive system and the radula, and a reference to the presence of a penial stylet, later confirmed by Marcus (1982). This is consistent with the characteristics of the specimens here assigned to *C. ocellifera*. Clark (1984) studied animals collected from near St. George's Harbor, Bermuda, the type locality of *Doto ocellifera*, and concluded that this species is in fact a member of *Costasiella* and a senior synonym of *Stiliger lilianae*. The original description of *D. ocellifera* (Fig. 7A) shows the distinctive eye structure of this species as well as other external characteristics summarized by Clark (1984). The specimens illustrated by Clark (1984) (see Fig. 7B) also match the original description of *Stiliger lilianae* and the characteristics of the animals here assigned to *C. ocellifera*.

More recently, Miles and Clark (2002) found different egg masses produced by specimens of *Costasiella ocellifera* from two different populations in the Florida Keys. These two populations have different developmental modes (planktotrophic and intracapsular metamorphosis). However, Miles and Clark (2002) could not find any morphological differences between members of the two populations. They proposed two hypothesis to explain their observations: poecilogony and the existence of two cryptic species. In a more comprehensive study, Ellingson (2006) showed that a single poecilogonous species was involved. *Costasiella patricki* n. sp. does not correspond to any of these populations in the Florida Keys. Patrick Krug (pers. comm.) has extensively

sampled populations of *C. ocellifera* in Florida and other Caribbean locations with different developmental modes, but has never collected or sequenced *C. patricki* n. sp. Other published records and illustrations of *C. ocellifera* from the Dominican Republic (Domínguez 2003), the Bahamas (Poddubetskaia 2004), the US Virgin Islands (Wilk 2005), Belize and Jamaica (Valdés *et al.* 2006), and Barbados (Alleyne 2006), clearly belong to *C. ocellifera*. The only unquestionable record of *C. patricki* n. sp. available in the literature is from the Bahamas. Redfern (2001, figs. 800A–D) illustrated the color variation of *C. ocellifera* in the Bahamas, and whereas fig. 681A corresponds to *C. ocellifera*, fig. 681B corresponds to *C. patricki* n. sp. In a second edition of this work, Redfern (2013) included additional photos showing the variability of *C. ocellifera*, but in this case none of them belong to *C. patricki*. Other published photos by Espinosa *et al.* (2006, fig. 415) from Cuba and Gundersen (2003) from Jamaica, could correspond to *C. patricki*, as they represent specimens with light green cerata bearing small orange rings and numerous black dots. However, this should be confirmed with molecular and morphological data.

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LITERATURE CITED

Akaike, H. 1974. A new look at the statistical model identifications. *Institute of Electrical and Electronics Engineers Transactions on Automatic Control* **19**: 716–723.

Alleyne, K. 2006. *Costasiella ocellifera* from Barbados. Available at: http://www.seaslugforum.net/find/16178 18 July 2013.

Baba, K. 1959. The family Stiligeridae from Japan (Opisthobranchia: Sacoglossa). *Publications of the Seto Marine Biological Laboratory* **7**: 327–334, pls. 27–28.

Baba, K. 1961. Three new species of the genus *Catriona* from Japan (Nudibranchia: Eolidacea). *Publications of the Seto Marine Biological Laboratory* **9**: 367–372, pls.14–15.

Baba, K. and I. Hamatani. 1970. Occurrences of specimens presumably identifiable with *Stiliger ornatus* Ehrenberg, 1831, at Seto, Kii, Middle Japan. *Publications of the Seto Marine Biological Laboratory* **18**: 199–206.

Cimino, G. and M. T. Ghiselin. 2009. Chemical defense and the evolution of opisthobranch gastropods. *Proceedings of the California Academy of Sciences* **60**: 175–422.

Clark, K. B. 1984. New records and synonymies of Bermuda opisthobranchs (Gastropoda). *The Nautilus* **98**: 85–97.

Colgan, D. J., A. McLauchlan, G. D. F. Wilson, S. P. Livingston, G. D. Edgecombe, J. Macaranas, G. Cassis, and M. R. Gray. 1998. Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. *Australian Journal of Zoology* **46**: 419–437.

Domínguez, E. 2003. *Costasiella ocellifera* from Dominican Republic. Available at: http://www.seaslugforum.net/find/9660 18 July 2013

Drummond, A. J., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. Moir, S. Stones-Havas, T. Thierer, and A. C. Wilson. 2010. Geneious version 4.8. Available at: http://www.geneious.com/web/geneious/geneious-versions 3 March 2010.

Ellingson, R. A. 2006. *Variable Development Versus Cryptic Speciation: Phylogeography and Evolutionary History of the Sea Slugs* Alderia *and* Costasiella *(Opisthobranchia: Sacoglossa)*. Master's Thesis, California State University Los Angeles, California.

Espinosa, J., J. Ortea, M. Caballer, and L. Moro. 2006. Moluscos marinos de la peninsula de Guanahacabibes, Pinar del Río, Cuba, con la descripción de nuevos taxones. *Avicennia* **18**: 1–84. [In Spanish].

Farris, J. S., M. Källersjö, A. G. Kluge, and C. Bult. 1994. Testing significance of incongruence. *Cladistics* **10**: 315–319.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294–299.

Gundersen, R. W. 2003. *Costasiella ocellifera* in Jamaica. Available at: http://www.seaslugforum.net/find/11110 18 July 2013

Hay, M. E., J. E. Duffy, V. J. Paul, P. E. Renaud, and W. Fenical. 1990. Specialist herbivores reduce their susceptibility to predation by feeding on the chemically defended seaweed *Avrainvillea longicaulis*. *Limnology and Oceanography* **35**: 1734–1743.

Huelsenbeck, J. P. and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.

Ichikawa, M. 1993. Saccoglossa (Opisthobranchia) from the Ryukyu Islands. *Publications of the Seto Marine Biological Laboratory* **36**: 119–139.

Jensen, K. R. 1985. Annotated checklist of Hong Kong Ascoglossa (Mollusca, Opisthobranchia) with descriptions of four new species. *In*: Morton, B. and D. Dudgeon, eds., *Proceedings of the Second International Workshop on the Malacofauna of Hong Kong and Southern China*. Hong Kong University Press, Hong Kong. Pp. 77–107.

Jensen, K. R. 1997. Sacoglossa (Mollusca, Opisthobranchia) from the Darwin Harbour area. Northern Territory, Australia. *In*: J. R. Hanley, G. Caswell, D. Megirian, and H. K. Larson, eds., *Proceedings of the Six International Marine Biological Workshop. The Marine Flora and Fauna of Darwin Harbour, Northern Territory, Australia*. Museums and Art Galleries of the Northern

Territory and the Australian Marine Sciences Association: Darwin, Australia. Pp. 163–186.

Masters, B. C., V. Fan, and H. A. Ross. 2011. Species delimitation: A Geneious plugin for the exploration of species boundaries. *Molecular Ecology Resources* **11**: 154–157.

Marcus, Ev. 1976. Marine euthyneuran gastropods from Brazil (3). *Studies on Neotropical Fauna and Environment* **11**: 5–23.

Marcus, Ev. 1977. An annotated check list of the western Atlantic warm water opisthobranchs. *Journal of Molluscan Studies* Supplement **4**: 1–22.

Marcus, Ev. 1982. Systematics of the genera of the order Ascoglossa (Gastropoda). *Journal of Molluscan Studies*, Supplement **10**: 1–31.

Marcus, Ev. and Er. Marcus. 1969. Euthyneure Meeresschnecken Brasiliens (2). *Beitrage zur Neotropischen Fauna* **6**: 1–16. [In German].

Mickevich, M. F. and J. S. Farris. 1981. The implications of congruence in Menidia. *Systematic Zoology* **30**: 351–370.

Miles, C. M. and K. B. Clark. 2002. Comparison of biochemical composition and developmental mode in two populations of *Costasiella* [Opisthobranchia: Ascoglossa (=Sacoglossa)]. *Journal of Molluscan Studies* **68**: 101–109.

Nylander, J. A. A. 2004. MrModeltest version 2. Available at: http://www.abc.se/~nylander 28 October 2010.

Ornelas-Gatdula, E., A. DuPont, and A. Valdés. 2011. The tail tells the tale: Taxonomy and biogeography of some Atlantic *Chelidonura* (Gastropoda: Cephalaspidea: Aglajidae) inferred from nuclear and mitochondrial gene data. *Zoological Journal of the Linnean Society* **163**: 1077–1095.

Palumbi, S. R. 1996. Nucleic acids II: The polymerase chain reaction. *In*: Hillis, D. M., C. Moritz, and B. K. Mable, eds., *Molecular Systematics*. Sinauer, Sunderland, Massachusetts. Pp. 205–247.

Posada, D. 2004. Collapse version 1.2. A tool for collapsing sequences to haplotypes. Available at: http://darwin.uvigo.es 9 May 2004.

Poddubetskaia, M. 2004. *Costasiella ocellifera* from Bahamas. Available at: http://www.seaslugforum.net/find/12276 18 July 2013

Puillandre, N., A. Lambert, S. Brouillet, and G. Achaz. 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* **21**: 1864–1877.

Rambaut, A. and A. J. Drummond. 2007. Tracer version 1.4. Available at: http://tree.bio.ed.ac.uk/software/tracer/ 28 October 2010.

Redfern, C. 2001. *Bahamian Seashells: A Thousand Species from Abaco, Bahamas*. Bahamianseashells.com, Inc., Boca Raton, Florida.

Redfern, C. 2013. *Bahamian Seashells: 1161 Species from Abaco, Bahamas*. Bahamianseashells.com, Inc., Boca Raton, Florida.

Simroth, H. 1895. Die Gastropoden der Plankton Expedition. *Ergebnisse der Plankton Expedition der Humboldt Stiftung* **2**(F.d.): 1–206, pls. 1–22. [In German].

Swennen, C. 2007. *Costasiella coronata*, new species, and a revised diagnosis for the family Costasiellidae (Mollusca: Opisthobranchia: Sacoglossa). *The Raffles Bulletin of Zoology* **55**: 355–362.

Swofford, D. L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods) Version 4. Sinauer, Sunderland, Massachusetts.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.

Thompson, T. E. 1977. Jamaican opisthobranch molluscs I. *Journal of Molluscan Studies* **43**: 93–140, pls. 1–3.

Valdés, A., J. Hamann, D. W. Behrens, and A. DuPont. 2006. *Caribbean Sea Slugs: A Field Guide to the Opisthobranch Mollusks from the Tropical Northwestern Atlantic*. Sea Challengers Natural History Books, Etc., Gig Harbor, Washington.

Wilk, L. 2005. *Costasiella ocellifera* from St. Vincent. Available at: http://www.seaslugforum.net/find/15054 18 July 2013

Xia, X. and P. Lemey. 2009. Assessing substitution saturation with DAMBE. *In*: P. Lemey, M. Salemi, and A.-M. Vandamme, eds., *The Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny*, 2nd Edition. Cambridge University Press, Cambridge, U.K. Pp. 615–630.

Xia, X. and Z. Xie. 2001. DAMBE: Data analysis in molecular biology and evolution. *Journal of Heredity* **92**: 371–373.

Xia, X., Z. Xie, M. Salemi, L. Chen, and Y. Wang. 2003. An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution* **26**: 1–7.

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